

# Circulating bovine pregnancy associated glycoproteins are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows<sup>1</sup>

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**ABSTRACT:** The objective was to examine the relationship between ovulatory follicle size and embryo and fetal survival by using circulating concentrations of bovine pregnancy associated glycoproteins (bPAG) to detect the presence of an embryo or fetus and monitor placental function. Before examining the relationship between bPAG, ovulatory follicle size, and embryo and fetal survival, the half-life of bPAG was determined in Exp. 1. The half-life of bPAG after PGF<sub>2α</sub>-induced abortion on d 32 to 36 postinsemination was 35.8 ± 21.9 h (mean ± SD; range 7.1 to 78.5 h). In Exp. 2, suckled beef cows (*n* = 91) were treated with the CO-Synch protocol (GnRH on d -9, PGF<sub>2α</sub> on d -2, and GnRH and AI 48 h later [d 0]) and classified into 1 of 2 ovulatory follicle size groups: 1) small follicle (<12.5 mm; *n* = 25) or 2) large follicle (≥12.5 mm; *n* = 66). The first increase (*P* < 0.0001) in serum bPAG occurred in pregnant cows on d 24 after insemination and circulating bPAG decreased before a decrease in progesterone in 3 of 4 cows that lost an embryo or fetus. Pattern of secretion of bPAG in serum from d 24 to 60 after insemination (d 0) was affected by day (*P* < 0.0001), but not ovulatory follicle size. In Exp. 3, suckled beef cows (*n* = 1164) were

administered the CO-Synch protocol either with (donor cows; *n* = 810) or without (recipient cows; *n* = 354) AI on d 0. Single embryos (*n* = 394) or oocytes (*n* = 45) were recovered from the donor cows [d 7; embryo transfer (ET)] and all live embryos were transferred into recipients the same day. Cows were classified on d 0 as having a small (<12.5 mm) or large (≥12.5 mm) ovulatory follicle, and randomly chosen as donors or recipients to remove confounding effects of ovulatory follicle size on fertility. Serum concentration of bPAG at d 28 was not affected by ovulatory follicle size (*P* = 0.85), embryo stage at ET (*P* = 0.75), embryo quality at ET (*P* = 0.64), estradiol at GnRH2 (*P* = 0.62) or serum progesterone at ET (d7; *P* = 0.14). Compared with cows that maintained pregnancy (*n* = 176), cows that exhibited late embryonic or fetal mortality (*n* = 19) after d 28 had decreased (*P* < 0.05) concentrations of bPAG on d 28. In summary, there was no relationship between serum bPAG and ovulatory follicle size or embryo stage or quality at ET; however, cows that lost an embryo after d 28 had reduced concentrations of bPAG on d 28 compared with cows that maintained pregnancy.

**Key words:** beef cow placenta, embryo survival, pregnancy

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## INTRODUCTION

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The GnRH-induced ovulation of small dominant follicles decreased pregnancy rates (Perry et al., 2005; Peres et al., 2009; Sa Filho et al., 2009) and increased late embryonic or fetal mortality in postpartum beef cows (Perry et al., 2005). In the preceding study, most of the embryonic or fetal mortality occurred around the time of embryo-uterine attachment (d 27 to 41) and may have been due to improper placentation. Piedrahita et al. (2002) reported conceptuses produced from so-

matic cell nuclear transfer of oocytes from small versus large bovine follicles (<3 versus 6 to 12 mm) developed smaller extraembryonic membranes ( $P < 0.002$ ). Consequently, the competence of oocytes ovulated from small dominant follicles may be compromised and negatively affect placentation. Furthermore, both oocyte competence (i.e., fertilization rate and embryo quality) and maternal environment (i.e., estradiol at d 0 and progesterone at d 7) were compromised after GnRH-induced ovulation of physiologically immature dominant follicles in postpartum beef cows (Atkins et al., 2013).

Bovine pregnancy associated glycoproteins (**bPAG**) are expressed in giant binucleate trophoblast cells (binucleate trophoblast cells) of the placenta, can be detected in the maternal circulation beginning around d 24 to 26 of gestation, and have been used to monitor embryonic or fetal viability as well as placental function in cattle (Sasser et al., 1986; Zoli et al., 1992; Green et al., 2000, 2005). We hypothesized that GnRH-induced ovulation of oocytes from small dominant follicles would result in decreased serum concentrations of bPAG and that cows that experience late embryonic or fetal loss (after d 28 of gestation) would have reduced circulating concentrations of bPAG on d 28 compared with cows in which pregnancy is maintained. The objectives were to determine the relationship between ovulatory follicle size and circulating concentrations of bPAG, and between circulating concentrations of bPAG and embryo and fetal survival in beef cows.

## MATERIALS AND METHODS

All protocols and procedures were approved by the University of Missouri Animal Care and Use Committee or by the Fort Keogh Livestock and Range Research Laboratory (LARRL) Animal Care and Use Committee.

### Animal and Treatments

**Experiment 1—Determination of Half-life of bPAG during Early Gestation.** Pregnancy specific protein B (or **bPAG1**) has a half-life of 7.3 d to 8.4 d after normal parturition (Ruder and Sasser, 1986; Kiracofe et al., 1993); consequently, this member of the pregnancy specific protein (**PAG**) family may not be particularly suitable for accurately determining the time of late embryonic or fetal loss. Therefore, an experiment was conducted to determine whether the bPAG measured by an ELISA (Green et al., 2005) during early gestation have a shorter half-life as suggested by Green et al. (2005). Estrus was synchronized in 38 nonpregnant, nonsuckled crossbred beef cows and heifers. The animals were artificially inseminated over a 5 d period. Pregnancy was diagnosed via transrectal ultrasonography postinsemi-

nation and pregnant cows ( $n = 25$ ) were allotted to a control ( $n = 10$ ) or PGF<sub>2α</sub> (25 mg as 5 mL i.m. of Lutalyse) treatment ( $n = 15$ ) group by age, breed, and days post AI on d 32 to 36 (mean = d 33) of gestation and observed for signs of estrus 3 times daily. The PGF<sub>2α</sub> was administered to the treatment cows on d 32 to 36 (mean d = 33) of gestation.

**Blood Sample Collection.** All blood samples (volume = 10 mL) were collected by using venipuncture and blood was allowed to clot at room temperature for 1 h, and at 4°C for 24 h. Samples were centrifuged at  $3,000 \times g$  for 20 min at 4°C, serum was decanted, and stored at -20°C until concentrations of bPAG were determined via ELISA (described below). Samples were collected every other day from d 20 to 28 post-AI, daily from d 29 to PGF<sub>2α</sub> injection, and every 8 h from PGF<sub>2α</sub> injection until serum concentrations of bPAG decreased to the basal concentrations established around the time of insemination.

**Ultrasonography.** In Exp. 1, the uteri of all cows were examined by transrectal ultrasonography at approximately d 28 after AI to verify the presence of a viable embryo (detection of heart beat). Ultrasonography was again performed in all cows just before the PGF<sub>2α</sub> injection and subsequently at 8-h intervals to monitor the presence and viability of the embryo. Time of embryonic death and loss of embryos from the uterus were estimated by the mean time from PGF<sub>2α</sub> to the ultrasound scans when the heartbeat or embryo was no longer present.

**Experiment 2—Effect of GnRH-induced Ovulatory Follicle Size on Serum Concentrations of bPAG.** Pregnant postpartum suckled (2- to 16-yr old) crossbred beef cows ( $n = 91$ ) at the University of Missouri-Columbia Beef Farm (yr 1:  $n = 47$ , mean days postpartum = 70.2; range 32 to 94 d; yr 2:  $n = 44$ , mean days postpartum 76.4; range 36 to 99 d) which conceived after treatment with the CO-Synch protocol: GnRH [100 µg as 2 mL intramuscular (**i.m.**) of Cystorelin, Merial], on d -9, PGF<sub>2α</sub> (25 mg as 5 mL i.m. of Lutalyse, Pfizer Animal Health) on d -2, and a second injection of GnRH (100 µg as 2 mL i.m.) 48 h after PGF<sub>2α</sub> on d 0 were used in this experiment. Cows were artificially inseminated by an experienced technician with semen from 1 of 2 bulls at the second GnRH injection (d 0) and there was no effect of bull on serum concentrations of bPAG. Treatment groups consisted of GnRH-induced ovulation (d 0) of these follicular sizes, based on previously reported follicle sizes in beef cows (Lamb et al., 2001; Perry et al., 2005): small follicle (<12.5 mm;  $n = 25$ ) or large follicle ( $\geq 12.5$  mm;  $n = 66$ ), and the study was conducted over a 2 yr period. Any cows that exhibited estrus [as determined by visual observation and activation of a KMAR (Steamboat Springs, CO) heat mount detector] before the second GnRH injection (d 0) were artificially inseminated

approximately 12 h after the onset of estrus and removed from the experiment. Calves were maintained with cows at all times and allowed to suckle without restriction. Cows were removed from the follicular size groups if they exhibited estrus before the second GnRH injection, did not ovulate in response to the GnRH injection at the time of insemination, or ovulated 2 follicles in response to the GnRH injection at the time of insemination.

**Blood Sample Collection.** All blood samples (volume = 10 mL) were collected via jugular or tail venipuncture into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA). Samples were collected on d -19 and -9 to determine estrous cycling status. Cows were considered anestrus if they had serum concentrations of progesterone fewer than 1 ng/mL at each of the preceding time points. Additional serum samples were also collected at PGF<sub>2α</sub> (d -2), GnRH (d 0), and d 7 after insemination. Serum was collected ( $n = 91$ ) every other day from d 20 to 60 during yr 1 and 2. In yr 2, additional blood samples were collected daily from d 26 to 30 ( $n = 44$ ) and monthly from d 60 until parturition. Blood was allowed to clot at room temperature for 1 h, and at 4°C for 24 h. Samples were centrifuged at  $3,000 \times g$  for 20 min at 4°C, serum was decanted, and stored at -20°C until concentrations of progesterone (P4), estradiol-17β (E2), and bPAG were determined via RIA (described below) or ELISA (bPAG, described below).

**Ultrasonography.** Ovaries of all animals were examined by transrectal ultrasonography to record follicular development by using an Aloka 500V ultrasound (Aloka, Wallingford, CT) with a 7.5 MHz transrectal linear probe. All follicles (>8 mm) were recorded on the day of PGF<sub>2α</sub> injection (all groups) and at the second GnRH injection (d 0). Transrectal ultrasonography was also performed 2 d after the GnRH injection (d 2) to verify ovulation of the largest follicle. Ovulation was defined by the disappearance of a dominant follicle from an ovary.

Pregnancy status was determined on d 28 to 31 after the second GnRH injection and continued every 2 wk until d 70 to 72 after GnRH for a total of 4 pregnancy exams per animal. An Aloka 500V ultrasound was used with a 7.5 MHz or 5.0 MHz transducer (depending on the stage of pregnancy) to determine presence of a fetus and fetal heartbeat.

**Experiment 3—Effect of Embryo Stage, Embryo Quality, and Embryonic and Fetal Survival on bPAG.** These data were collected as part of a larger study that was reported by Atkins et al. (2013). Experimental design, treatments, and some of the methodologies were originally described by Atkins et al. (2013) and modified for inclusion in this publication. This experiment occurred over 3 yr with 9, 11, and 5 replicates of suckled postpartum beef cows in yr 1, 2, and 3, respectively. This study includes data on 1164 single ovulations

in suckled beef cows that were synchronized with the CO-Synch protocol (GnRH) on d -9 followed by PGF<sub>2α</sub> (d -2) and GnRH on d 0 with fixed-timed AI) of which 810 were donor cows (inseminated on d 0) and 354 were recipient cows (not inseminated). Donor cows were inseminated by 1 of 3 AI technicians with semen from a single sire. Single embryos were recovered by uterine horn lavage 7 d after the second GnRH and all live embryos were transferred fresh into recipients on the same day as recovery. Cows were classified on d 0 as having a small (<12.5 mm) or large (≥12.5 mm) ovulatory follicle and randomly chosen as donors or recipients to remove confounding effects of ovulatory follicle size on fertility.

**Blood Sample Collection.** All blood samples were collected by using venipuncture and processed as described in Exp. 2, with the exception that Exp. 3 included sampling of both recipient and donor animals. Blood samples were collected from cows diagnosed pregnant (visible fetal heartbeat), on d 27–29, d 40–42, d 54–56, and d 70–72. Cows not maintaining pregnancy were bled 1 time after a nonpregnant diagnosis before removal from the study.

**Ultrasonography.** Real-time ultrasonography was conducted on donor and recipient cows as described in Exp. 2.

### **Embryo Recovery and Transfer**

The following embryo recovery and transfer protocol was used in Exp. 3 and previously described by Atkins et al. (2013). As mentioned earlier, cows were classified on d 0 as having a small (<12.5 mm) or large (≥12.5 mm) ovulatory follicle and randomly chosen as donors or recipients to remove confounding effects of ovulatory follicle size on fertility. Embryos were recovered from donor cows by using a nonsurgical transcervical uterine catheterization technique on d 7 (Atkins et al., 2013). Because these were all single embryo recoveries, the catheters were placed in the uterine horn ipsilateral to the corpus luteum (CL) and a single horn was lavaged with ViGro Complete Flush Solution (Bioniche Animal Health, Athens, GA). Embryos were transferred fresh into the uterine horn ipsilateral to the CL of recipient cows on the same day as collection by 1 of 2 technicians. Embryos collected from cows that ovulated a small follicle were transferred into cows that ovulated a large ( $n = 111$ ) or small ( $n = 71$ ) follicle. Similarly, embryos collected from cows that ovulated a large follicle were transferred into cows that ovulated a large ( $n = 50$ ) or small ( $n = 122$ ) follicle.

### **Embryo Handling**

The following methods for handling embryos in Exp. 3 were previously described (Atkins et al., 2013). Em-



bryos and oocytes were washed 3 times in holding media (Biolife Holding Media, AgTech Inc., Manhattan, KS) and stored at 26°C until grading and transfer. Embryo development [scale of 1 to 7 where 1 = unfertilized oocyte (UFO) and 7 = expanded blastocyst] and quality [scale of 1 to 4 where 1 = excellent to good (85% of the cellular mass was intact and healthy in appearance), 2 = fair (between 50 and 85% of the cellular mass was intact and healthy in appearance and no abnormalities in embryo shape), 3 = poor (over 50% of the cellular mass is extruded or degenerating or gross abnormalities in the structure of the embryo), and 4 = degenerate or dead; based on the classifications set by the International Embryo Transfer Society (Savoy, IL)] were determined. All live embryos (quality grades 1 to 3) were transferred into recipients except embryos that were lost or damaged before transfer ( $n = 8$ ) or did not have a recipient ( $n = 2$ ).

### Assays

Serum concentrations of P4 were quantified by RIA with a Coat-a-Count RIA kit (Diagnostic Products Corporation, Los Angeles, CA) as described previously (Bellow et al., 1991; Kirby et al., 1997). Intra- and inter-assay CV were 2.5 and 11% (Exp. 1), and 1.8 and 13% (Exp. 3), respectively, and the assay sensitivity was 0.08 ng/mL for the progesterone RIA. Serum concentrations of E2 were measured by using RIA (Kirby et al., 1997) in samples collected on the day of insemination. The intra- and interassay CV for the E2 RIA were 9.5 and 18.8% (Exp. 2), and 2.9 and 14% (Exp. 3), respectively, and the assay sensitivity was 0.05 pg/mL. Concentrations of bPAG in serum were determined by a monoclonal-based bPAG ELISA as described by Green et al. (2005) and the assay sensitivity was 0.28 ng/mL. Each assay included a standard curve, a pooled sample from pregnant cows d 60 of gestation, and a pooled sample from nonpregnant cows and the assay sensitivity was 0.28 ng/mL of bPAG. Samples with concentrations of bPAG below assay sensitivity were considered as 0.28 ng/mL in all data analysis. The intra- and interassay CV for the bPAG ELISA were 7.1 and 14.7% across all experiments.

### Statistical Analysis

Effect of ovulatory follicle size, d 0 E2 concentration, and d 7 P4 concentration on serum concentrations of bPAG were analyzed by ANOVA for repeated measures (Proc Mixed; Littell et al., 1998; SAS Inst. Inc., Cary, NC). The statistical model consisted of the variable tested, day, and their interactions. Analysis of breakpoints was conducted by using Proc NLIM (Robbins et al., 2006) in SAS, and was used to determine the first significant change in the slope of the line (secretion

of bPAG). Simple correlations were also determined in SAS. Half-life of bPAG, after loss of embryonic heart-beat, was determined by using this formula:  $t_{1/2} = \ln 2/k$  (Kiracofe et al., 1993), where  $k$  is the slope of the regression equation, which was determined by GLM procedures of SAS. The effect of embryo quality and embryo stage on bPAG concentrations was determined by using a PROC MIXED model in SAS. One-way ANOVA (SAS) was used to test differences among d 28 serum concentrations of bPAG for postpartum beef cows that maintained pregnancy and those that established but did not maintain pregnancy. The probability of pregnancy maintenance based on d 28 bPAG concentrations was modeled by logistic regression using the LOGISTIC procedure in SAS.

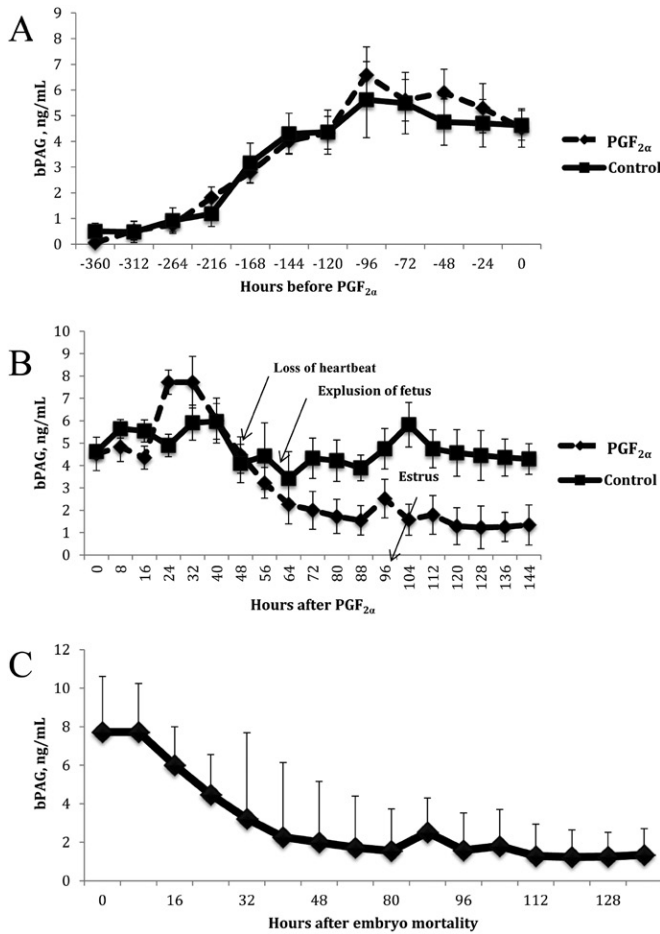
## RESULTS

### Experiment 1—Determination of Half-Life of bPAG during Early Gestation

Serum concentrations of bPAG in the control and PGF<sub>2α</sub> cows increased similarly until PGF<sub>2α</sub> treatment (Fig. 1a). At PGF<sub>2α</sub> treatment (0 h), mean ( $\pm$ SEM) serum concentrations of bPAG for control ( $n = 10$ ) and PGF<sub>2α</sub> treated cows ( $n = 15$ ) were  $4.62 \pm 0.94$  ng/mL (range 2.66 to 8.23 ng/mL) and  $4.52 \pm 0.39$  ng/mL (range 1.04 to 12.55 ng/mL), respectively. After PGF<sub>2α</sub> treatment, embryonic mortality ( $n = 15$ ), expulsion of fetus from the uterus ( $n = 15$ ), and return to estrus ( $n = 14$ ) occurred within  $47.2 \pm 9.9$  h,  $57.3 \pm 9.9$  h, and  $93.1 \pm 12.1$  h, respectively (Fig. 1b). After embryonic mortality, the half-life of circulating concentrations of bPAG was  $35.8 \pm 21.9$  h (mean  $\pm$  SD; range 7.1 to 78.5 h; Fig. 1c).

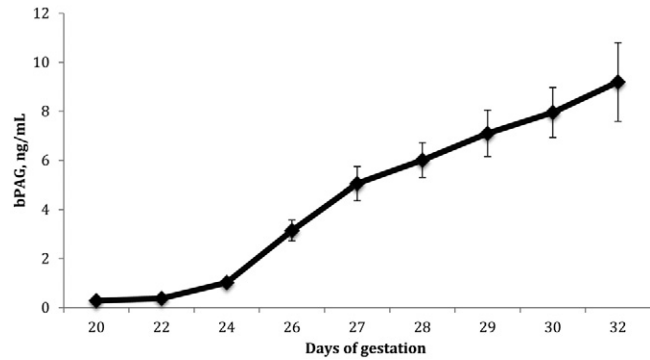
### Experiment 2—Effect of GnRH-Induced Ovulatory Follicle Size on Serum Concentrations of bPAG

Mean ( $\pm$ SD) ovulatory follicle size was  $14.24 \pm 1.96$  mm and there was no follicle by year interaction ( $P = 0.41$ ); therefore, the data from yr 1 and 2 were combined. Serum concentrations of bPAG in pregnant cows (mean  $\pm$  SEM =  $1.02 \pm 0.10$  ng/mL; range = 0.28 to 4.13 ng/mL) was increased ( $P < 0.01$ ) on d 22 compared with nonpregnant cows ( $0.29 \pm 0.05$  ng/mL). Alternatively, the first increase ( $P < 0.0001$ ) in bPAG, based on breakpoint analysis, occurred on d 24 (Fig. 2). There was 100% agreement between pregnancy diagnosis by real-time ultrasonography and increased serum concentrations of bPAG on d 28. The pattern of secretion of bPAG increased from d 24 to approximately d 36 of gestation and subsequently declined (Fig. 3a). The serum concentration of bPAG from d 24 to 60 after insemination (d 0) was not affected by ovulatory follicle size ( $P =$



**Figure 1.** Serum concentrations of bovine pregnancy associated glycoproteins (bPAG; Panel A; mean  $\pm$  SEM) before PGF<sub>2α</sub> treatment ( $n = 15$ ; d 33) or no treatment (Control,  $n = 10$ ; d 32 to d 36), (Panel B) after PGF<sub>2α</sub> treatment or control and (Panel C) after embryonic mortality (0 h; loss of heartbeat) in cows from Exp. 1. Circulating concentrations of bPAG increased similarly until PGF<sub>2α</sub> treatment (injection of prostaglandin F<sub>2α</sub>) with a mean  $\pm$  SEM bPAG concentration for the control group of  $4.62 \pm 0.94$  ng/mL (range 2.66 to 8.23 ng/mL) and for the PGF<sub>2α</sub> group of  $4.52 \pm 0.39$  ng/mL (range 1.04 to 12.55 ng/mL). After PGF<sub>2α</sub> injection (0 h) embryonic mortality as determined by loss of heartbeat ( $n = 15$ ), expulsion of fetus from uterus ( $n = 15$ ), and return to estrus ( $n = 14$ ) occurred at  $47.2 \pm 9.9$  h,  $57.3 \pm 9.9$  h, and  $93.1 \pm 12.1$  h (mean  $\pm$  SD) after PGF<sub>2α</sub> administration, respectively. The half-life of bPAG was calculated to be  $35.8 \pm 21.9$  h (mean  $\pm$  SD; range 7.1 to 78.5 h).

0.96) or year ( $P = 0.90$ ). However, there was an effect of day on serum concentration of bPAG ( $P < 0.0001$ ). In yr 2, the pattern of secretion of bPAG increased monthly from mo 3 of gestation to calving and there was an effect of month but no effect of ovulatory follicle size or follicle size by month interaction on the secretion pattern of bPAG (Fig. 3b). Mean serum concentrations of bPAG on d 28 was  $6.17 \pm 5.24$  ng/mL (mean  $\pm$  SD; range 0.28 to 33.09 ng/mL) for all cows and there was no relationship ( $P = 0.54$ ) between ovulatory follicle size at breeding and serum concentrations of bPAG on d 28, progesterone on d 7 post AI ( $P = 0.96$ ) or progesterone on d 28 post AI ( $P = 0.31$ ). Similarly, there was no correlation



**Figure 2.** Serum concentrations of bovine pregnancy associated glycoproteins (bPAG) in pregnant cows (mean  $\pm$  SEM;  $n = 91$ ) from d 20 to d 32 of gestation in Exp. 2. First significant increase ( $P < 0.0001$ ) in circulating bPAG concentrations occurred on d 24 of gestation.

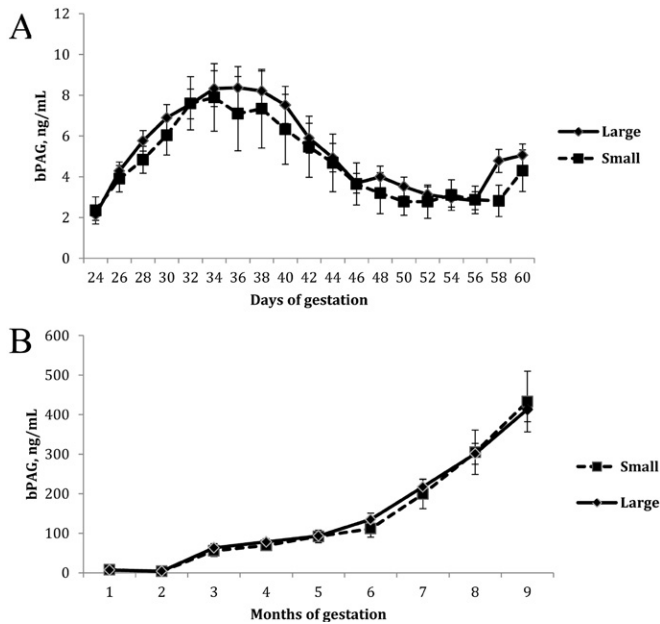
between serum estradiol at d 0 (AI) and serum bPAG at d 28 in pregnant cows ( $P = 0.38$ ).

There were 4 cows that experienced late embryonic or early fetal mortality between d 28 and 42 based on ultrasound, and in 3 of the 4 cows, serum concentrations of bPAG decreased before there was a decrease in serum concentration of progesterone, in the fourth cow, the decrease in bPAG and progesterone occurred simultaneously (Fig. 4).

### Experiment 3—Effect of Embryo Stage, Embryo Quality, and Embryonic and Fetal Survival on bPAG

Ovulatory follicle diameter was similar in both the donor and recipient cows with a mean ( $\pm$  SD) follicle diameter of all cows on d 0 (donor = AI; recipient = No AI) of  $12.5 \pm 1.8$  mm (range 8.05 to 18.55 mm). Serum concentration of bPAG from pregnant recipient cows did not differ ( $P = 0.85$ ) among embryo transfer groups [small to small (S-S;  $n = 71$ ), small to large (S-L;  $n = 111$ ), large to small (L-S;  $n = 122$ ) and large to large (L-L;  $n = 50$ )] from d 28 to d 72 of gestation. Neither serum concentrations of estradiol (donors,  $P = 0.62$ ; recipients,  $P = 0.43$ ) at d 0 nor progesterone on d 7 (donors,  $P = 0.14$ ; recipients,  $P = 0.10$ ) were correlated with serum bPAG on d 28 in pregnant recipient cows. Also, there was no effect of embryo quality at ET ( $P = 0.64$ ) or embryo stage at ET ( $P = 0.75$ ) on serum bPAG from d 28 to d 72 of gestation. In addition, donors ovulating to GnRH on d -9 resulted in significantly greater serum concentrations of bPAG in recipients on d 28 ( $P < 0.05$ ) and recipients not cycling at GnRH on d -9 resulted in significantly greater serum concentrations of bPAG on d 28 ( $P < 0.0001$ ).

In Exp. 3, bPAG were 98% accurate in diagnosing pregnancy on d 28 compared with real-time ultrasonography. There were 2 cows that had a viable embryo based on ultrasound; however, no bPAG were detected from d 28 to 72 of gestation. On d 27 to 29 there were 195 cows con-

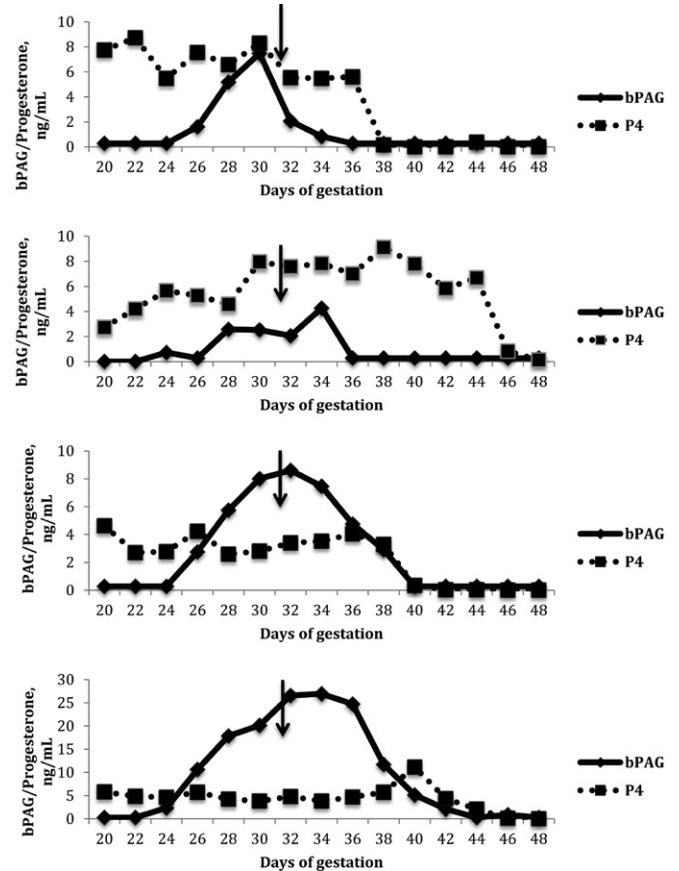


**Figure 3.** Effect of ovulatory follicle size, after GnRH-induced ovulation (d 0), on serum concentrations of bovine pregnancy associated glycoproteins (bPAG; mean  $\pm$  SEM) from d 24 to d 60 (Panel A; yr 1 and 2,  $n = 91$ ) and from mo 1 to 9 of gestation (Panel B; yr 2,  $n = 44$ ) from Exp. 2. Treatment groups included small ( $<12.5$  mm;  $n = 25$ ), or large ( $\geq 12.5$  mm;  $n = 66$ ) ovulatory follicles. Pattern of secretion of bPAG in serum from d 24 to 60 after insemination (d 0) was affected by day ( $P < 0.0001$ ), but not ovulatory follicle size ( $P = 0.96$ ) and there was no day and ovulatory follicle size interaction ( $P = 0.41$ ).

firmed pregnant via transrectal ultrasound, and by d 70 to 72 of gestation 19 (9.7%) animals had undergone late embryonic or early fetal mortality. The majority of these cows experienced embryonic mortality between d 27 to 40 ( $n = 10$ ) followed by d 40 to 56 ( $n = 7$ ) and d 56 to 72 ( $n = 2$ ; Fig. 5). Although all cows had an embryo with a heartbeat on d 28, mean serum concentrations of bPAG ( $P < 0.05$ ) were significantly greater in cows that maintained pregnancy ( $4.53 \pm 0.34$  ng/mL; mean  $\pm$  SEM) until d 72 compared with cows that experienced embryonic mortality ( $3.14 \pm 0.72$  ng/mL) at any point between d 28 to d 72 of gestation (Fig. 6). In addition, d 28 bPAG were included as a continuous variable in a logistical regression of pregnancy maintenance until d 72. As d 28 bPAG concentrations increased, there tended to be an increase ( $P < 0.10$ ) in the likelihood of maintaining a pregnancy until d 72 (Fig. 7). However, the bPAG concentration at d 28 was most accurate at predicting pregnancy failure by d 40 of gestation. Serum concentrations of progesterone on d 28 were similar in cows that maintained pregnancy to d 72 and animals that experienced embryonic mortality.

## DISCUSSION

Detection of PAG in the maternal circulation has been used to accurately diagnose pregnancy in ruminants, including cattle, sheep, goats, buffalo, bison, moose, and elk (Sass-

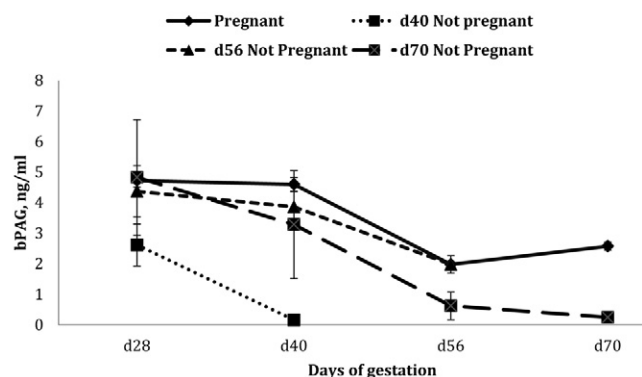


**Figure 4.** Serum concentrations of bovine pregnancy associated glycoproteins (bPAG) and progesterone (P4) for individual cows that experienced late embryonic or fetal mortality between d 30 and 45 of gestation (d 0 = AI) from Exp. 2. In 3 of the 4 cows, serum concentrations of bPAG decreased before a decrease in progesterone was detected. Arrows represent the last confirmed heartbeat of each embryo.

er et al., 1986; Sousa et al., 2006; Szafranska et al., 2006). In this study, bPAG were 99.2% accurate in detecting pregnancy by d 28 compared with real-time ultrasonography in beef cattle, and the first significant increase in bPAG occurred between d 22 and 24 of pregnancy. This time period corresponds to adhesion of the trophoblast to the endometrium and is preceded by the appearance of binucleate trophoblast cells (d 19 to 21; Wooding, 1983). The PAG enter the maternal circulation via exocytosis of secretory granules from binucleate trophoblast cells toward the maternal vasculature. Circulating concentrations of bPAG increased rapidly from d 24 until approximately d 36 and subsequently decreased until d 60. The physiological basis for the transient increase in bPAG during this time frame is not known. The rapid increase in bPAG during the last trimester may be due to the relatively rapid increase in placental size or release of sequestered PAG from various tissues (Green et al., 2005).

A limitation to the detection of bPAG for pregnancy diagnosis has been the relatively long half-life of pregnancy specific protein B (PSPB or PAG1; Ruder and Sasser, 1986; Semambo et al., 1992). Pregnancy specific protein B has been the primary PAG for detection

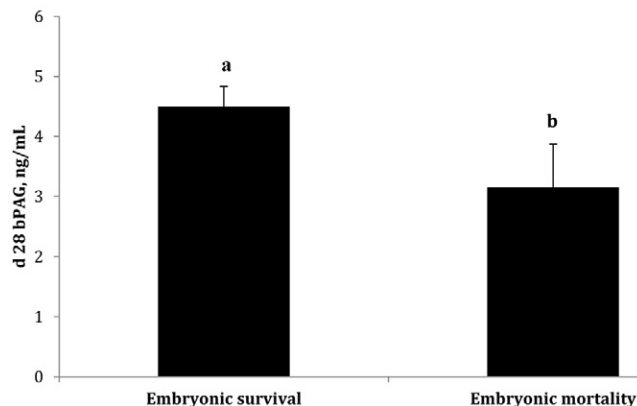




**Figure 5.** Serum concentrations of bovine pregnancy associated glycoproteins (bPAG) for cows that maintained pregnancy until d 72 (Pregnant;  $n = 176$ ) compared with cows that experienced late embryonic or fetal mortality (Not pregnant) between d 28 to 40 ( $n = 10$ ), d 40 to 56 ( $n = 7$ ), and d 56 to 72 ( $n = 2$ ) of gestation.

of pregnancy in ruminants (Sasser et al., 1986; Zoli et al., 1992). However, Green et al. (2000) proposed that a disadvantage to measuring bPAG1 for pregnancy detection is the long half-life (~8 d; Sasser et al., 1986) in the maternal circulation after parturition or fetal loss can result in false positives in early postpartum cows. Consequently, effort has been directed toward identifying bPAG with a shorter half-life. Green et al. (2005) established an ELISA that measures bPAG with a relatively short half-life (i.e., 4.3 d in the postpartum period). In the current study, the half-life of bPAG after induction of embryonic mortality was 1.5 d, which was considerably shorter than the half-life of previously reported bPAG. In a similar study, bPAG1 had a half-life of 7 d after induction of embryonic mortality in cows (Semambo et al., 1992) and even longer (7.3 to 8.4 d) after normal parturition (Ruder and Sasser, 1986; Kiracofe et al., 1993). Interestingly, Szenci et al. (2003) reported that the half-life of bPAG1 after embryonic mortality in heifers, was 2.7 to 3.9 d. Differences in half-life among studies might be due to detection of different members of the PAG family rather than a change in half-life during the course of gestation; however, this hypothesis has not been tested.

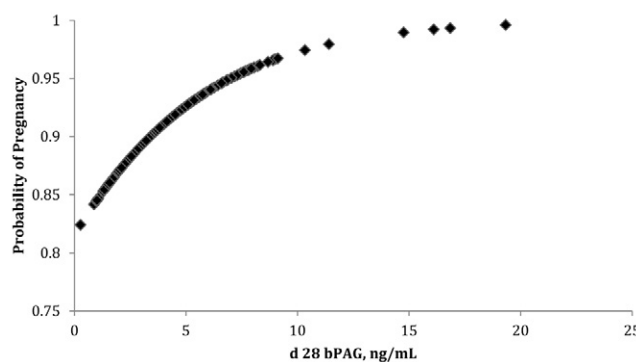
The GnRH-induced ovulation of small dominant bovine follicles (CO-Synch protocol) decreased pregnancy rate and increased late embryonic and fetal mortality (39%) from d 27 to 68 after insemination (Perry et al., 2005). In the present study, the majority of late embryonic or fetal mortality occurred around the time of embryo-uterine attachment (d 21 to 42; Peters 1996) and reproductive loss during this time has been reported by others (Vasconcelos et al., 1999; Cartmill et al., 2001; Moreira et al., 2001; Perry et al., 2005). Identification of a reliable marker of conceptus viability in the maternal circulation would be helpful in studying mechanisms by which the physiological maturity of an ovulatory follicle might affect late embryonic and fetal mortality. In Exp. 2, it was



**Figure 6.** Serum concentrations of bovine pregnancy associated glycoproteins (bPAG; mean  $\pm$  SEM) in postpartum beef cows that had a viable embryo on d 28 ( $n = 195$ ) of gestation and either maintained pregnancy (Embryonic survival;  $n = 176$ ) or did not maintain pregnancy (Embryonic mortality;  $n = 19$ ). Cows that experienced late embryonic or fetal mortality by d 72 had decreased serum concentrations of bPAG on d 28 compared with cows that maintained an embryo until d 72 of gestation ( $P < 0.05$ ).

not possible to precisely determine when embryonic death occurred relative to luteolysis; however, serum bPAG decreased before or at the same time as progesterone in the 4 cows that experienced late embryonic or fetal mortality. Similarly, Giordano et al. (2012) reported a relatively rapid decrease in bPAG and PSPB after embryonic death and PGF<sub>2 $\alpha$</sub> -induced luteolysis in pregnant dairy cows. These findings support the concept that death of the embryo or fetus, based on absence of a heartbeat, preceded luteolysis in cattle (Kastelic et al., 1991). Similarly, prolongation of luteal lifespan in beef heifers did not increase pregnancy rate (Wiltbank et al., 1961).

Mechanisms associated with late embryonic or fetal mortality after GnRH-induced ovulation of small dominant follicles in cattle may be due to inadequate oocyte competence (Lonergan et al., 2003), reduced luteal progesterone secretion (Inskeep, 2004; Diskin and Morris, 2008), inadequate uterine environment (Barnes, 2000), and/or in-



**Figure 7.** Probability of pregnancy maintenance until d 72 of gestation based on d 28 circulating concentrations of bovine pregnancy associated glycoproteins (bPAG;  $n = 195$ ) in lactating beef cows. Increased serum concentrations of bPAG on d 28 tended to increase the probability of pregnancy maintenance ( $P < 0.10$ ).

adequate placental function (Facciotti et al., 2009). Atkins et al. (2013) reported that decreased pregnancy success after GnRH-induced ovulation of small dominant follicles in postpartum beef cows may be due to inadequate oocyte competence as determined by decreased fertilization rate and a reduced probability of recovering a transferrable embryo. Inadequate cytoplasmic or nuclear maturation of an oocyte can reportedly compromise subsequent embryonic development and the establishment of pregnancy (Sirard et al., 2006; Pohler et al., 2012). Because oocyte competence may subsequently affect placental function (Piedrahita et al., 2002) we hypothesized that GnRH-induced ovulation of small dominant follicles may result in conceptuses that secrete less bPAG into the maternal circulation. However, there was no effect of ovulatory follicle size or ovulatory follicle size  $\times$  time interaction on circulating concentrations of bPAG from d 20 to 60 after insemination in the present study.

Another reason for decreased pregnancy success after GnRH-induced ovulation of small dominant follicles in postpartum beef cows may be an inadequate maternal environment resulting from decreased serum estradiol at GnRH-induced ovulation and/or decreased progesterone production from the resulting CL (Atkins et al., 2013; Jinks et al., 2013). Preovulatory secretion of E2 (Moore, 1985; Jinks et al., 2013) and postovulatory secretion of P4 (Mann and Lamming, 1999; Perry et al., 2005) have important roles in the establishment of pregnancy in cattle. However, in Exp. 2 and 3 serum bPAG were not affected by serum estradiol on d 0 or serum P4 at d 7 or 28.

Incidence of late embryonic or fetal mortality (after d 28) is approximately 4 to 10.8% in beef cattle and can be greater in dairy cattle (Inskeep, 2002; Lamb, 2002; Stevenson et al., 2003). Causes of late embryonic or fetal mortality are variable but include GnRH-induced ovulation of small (physiologically immature) dominant follicles (Perry et al., 2005). In the preceding study, cows not maintaining pregnancy until d 70 of gestation tended to have decreased circulating concentrations of bPAG on d 30, suggesting that these animals could potentially experience embryonic or fetal mortality due to inadequate placental function. In Exp. 3, cows destined to undergo late embryonic or early fetal loss by d 72 had significantly decreased bPAG on d 28 of gestation even though all cows had a viable fetus, based on the presence of a heartbeat. Breukelman et al. (2012) conducted a study to examine whether pregnancy loss after embryo transfer in cattle was affected by maternal concentrations of progesterone (before or after embryo transfer) and bPAG-1 (d 25 to 35 of gestation). Late embryonic or fetal mortality was not associated with circulating concentrations of progesterone; however, 4 different patterns of bPAG1 secretion were identified in cows that experienced pregnancy loss between d 26 and 120. Furthermore, concentrations

of bPAG 1 were reduced in cows that lost their pregnancy compared with cows in which pregnancy was maintained. These data suggest that serum bPAG at d 28 may be used to diagnosis pregnancy establishment and predict embryonic or fetal survival until at least d 72 of gestation, which accounts for the majority of embryonic or fetal mortality.

Circulating concentrations of bovine PAG can be influenced by a number of factors including breed, BW, parity status of the dam, fetal sex, fetal number, and fetal birth weight, along with stage of pregnancy (Patel et al., 1997; Perry et al., 2005; Lobago et al., 2009). Based on the positive association between serum bPAG on d 28 and embryonic or fetal survival, we examined the relationship between measures of embryonic development and quality at the time of embryo recovery or transfer and serum bPAG on d 28. However, there was no relationship between embryo quality or stage of embryonic development at embryo transfer and serum bPAG on d 28.

In summary, there was no relationship between serum bPAG and ovulatory follicle size, estradiol on d 0, progesterone on d 7 or d 28, and embryo quality or embryo stage on d 7 in the present study. However, cows that experienced embryonic or fetal mortality after d 28 had lower concentrations of bPAG on d 28 compared with cows that maintained pregnancy. Therefore, bPAG around d 28 may be a useful predictor of future embryonic or fetal mortality during early gestation.

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